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Patentanmeldung Nr.	Patent application No.	Demande de brevet n°
	00110084.1	

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

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THE HAGUE, 06/03/04
LA HAYE, LE

Anmeldung Nr:
Application no.: 00110084.1
Demande no:

Anmeldetag:
Date of filing: 12.05.00
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Anmelder/Applicant(s)/Demandeur(s):

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Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Radiometal labelled molecules having improved biological properties and method for preparation thereof

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s) revendiquée(s)
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/00.00.00/

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AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE

RADIOMETAL LABELLED MOLECULES HAVING IMPROVED BIOLOGICAL PROPERTIES AND METHOD FOR PREPARATION THEREOF

The present invention relates to a convenient synthesis of novel bifunctional prochelators for coupling to bioactive peptides for radiometal labelling and to the rediometal labelled peptides that can be prepared while using these novel prochelators.

The coupling of chelators to bioactive peptides requires the synthesis of prochelators which are compatible with solid and solution phase peptide synthesis.

According to the invention bifunctional macrocyclic synthons (prochelators) are provided based on DOTA, TRITA, TETA or structures comprising not 4, but 5 or 6 N atoms, which synthons are differentially protected and compatible with solid phase peptide synthesis procedures for labeling with hard Lewis acid radiometals.

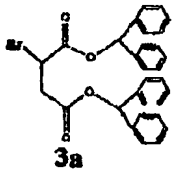
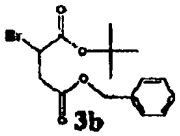
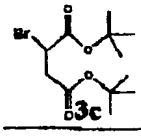
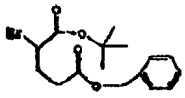
Starting from a relevant amino acid, the α-bromo-derivative thereof is synthesized. This derivative is orthogonally protected (tBu, Bzl). This alkylating agent will be reacted with cyclen, cyclam etc. to form a 1:1 adduct followed by tris-alkylation with bromoacetic acid tert-butylester and catalytic hydrogenation with H_2/Pd.

The synthon is monoreactive, carrying a free carboxylate group for coupling to the N-terminal end of the peptide and can be couled to any biomolecule which after deprotection can be labelled with a multitude of radiometals.

The following pages describe the synthesis of the prochelators in more detail.

Abstract: New DOTA-based bifunctional prochelators, e.g. 1-(1-carboxy-3-carbotertbutoxypropyl)-4,7,10-(carbotertbutoxymethyl)-1,4,7,10-tetraazacyclododecane (DOTAGA(tBu)$_4$, (6d) for a broad application in the modification of biomolecules with metal ions were prepared. The 5 step synthesis of 6d has an overall yield of about 20%. The coupling of 6d to a bioactive peptide on solid phase was exemplified with use of a CCK-B (cholecystokinin) analogue.

Table 1: Mono-alkylation yields of cyclen with different bromo-dicarboxylic acid diesters

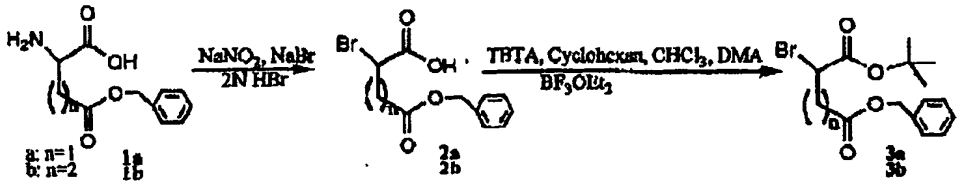
alkyl. agent	yield
 3a	79%
 3b	21%
 3c	<5%
 3d	83%

DOTA (1,4,7,10-tetrakis (carboxymethyl)-1,4,7,10 tetraazacyclo dodecane) and its derivatives constitute an important class of chelators for biomedical applications as they accomodate very stably a variety of di- and trivalent metal ions. Gd(DOTA)$^{-1}$ is an important MRI (Magnetic Resonance Imaging) contrast agent and as bifunctional versions DOTA is used in radioimmunotherapy[2].

An emerging area is the use of chelator conjugated bioactive peptides for labeling with radiometals in different fields of diagnostic and therapeutic nuclear oncology[3]. For their convenient and high yield synthesis prochelators (compounds which become chelators upon deprotection) are necessary which are compatible with the solid and solution phase peptide synthethic procedures.

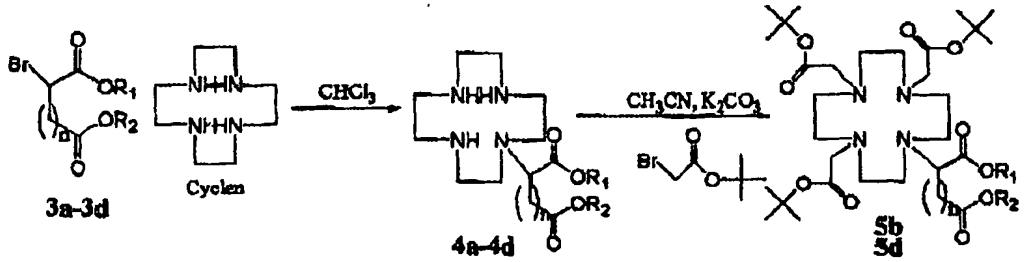
We describe herein the synthetic steps towards bifunctional orthogonally protected prochelators for coupling to the *N*-terminus of bioactive peptides or other useful amino functions in biomedical applications. The DOTA-derived chelator should provide 4 intact carboxylic acid functions besides the macrocyclic tetraazacyclododecane ring for a stable and efficient binding of metal ions and a function for biomolecule coupling.

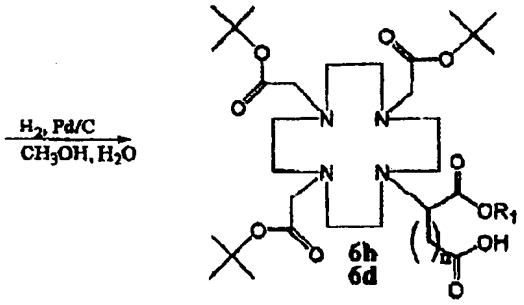
The strategy included the synthesis of an orthogonally protected bromo-alkyl-dicarboxylic acid diester for the monoalkylation of cyclen (1,4,7,10-tetraazacyclododecane). High yield monoalkylation of cyclen was demonstrated before[3,4,5]. The synthesis of 6 (n=1,2) is a 5 step procedure starting from the commercially available aspartic (1b) or glutamic acid-4-(5) benzyl ester (1d) (Scheme1) using a method analogeous to Holmberg[6] followed by *tert*-butylation using *tert*-butyltrichloroacetimidate (TBTA) as reagent[7,8].



Scheme1: Synthesis of α-bromosuccinic acid-1-*tert*butylester-4-benzyl ester (3b) and α-bromoglutaaric acid-1-*tert*butyl ester-5-benzyl ester (3d).

The monoalkylation of cyclen, the crucial step, showed strongly differing yields depending on the bromo-alkyl-dicarboxylic acid diester (3a-d) used (Table1). In earlier studies our strategy was to use metals as protecting groups[9]. In that work we attempted to introduce succinic acid-di-*tert*-butylester(3c) and found yields below 5% for the monoalkylation with the elimination product fumaric acid-di-*tert*-butylester as the main product. Interestingly the corresponding diphenylmethyl diester (3a) gave high monoalkylation yields and negligible elimination. With the homologous 2-bromoglutaric-1-*tert*butyl-5-benzylester (3d), no elimination product was found, obviously because no conjugated π-system could be formed. The remaining nitrogens were alkylated by use of three equivalents of bromoacetic acid-*tert*butyl ester in $CHCl_3/K_2CO_3$. Deprotection of the benzyl ester group was performed with H_2/ Pd/ C.





a: R_1; R_2=Bzh; n=1
b: R_1=tBu; R_2=Bn; n=1
c: R_1;R_2=tBu; n=1
d: R_1=tBu; R_2=Bn; n=2
Bzh: diphenylmethyl-

Scheme2: Synthesis of DOTASA(tBu)$_4$(6b) and DOTAGA(tBu)$_4$(6d).

The overall yield of 1-(1-carboxy-3-carbo*tert*butoxypropyl)-4,7,10-(carbo*tert*butoxymethyl)-1,4,7,10-tetraazacyclododecane (DOTAGA(tBu)$_4$) (6d) over 5 steps was about 20%[10] and of 1-(1-carboxy-2-carbo*tert*butoxyethyl)-4,7,10-(carbo*tert*butoxymethyl)-1,4,7,10-tetraazacyclododecane (DOTASA(tBu)$_4$) (6b) only about 2%. The convenient use of 6d is exemplified by its coupling to the CCK-B analogue D-Asp-Tyr-Nle-Gly-Trp-Nle-Asp-Phe-NH_2 (7) attached to Rink-amide resin using HATU (O-(7-azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluroniumhexafluorophosphate) as coupling reagent. After deprotection, (18h, rt, TFA:phenol:thioanisol:water 85:5:5:5) DOTAGA-7 was obtained in high yield[11] and showed superior properties in comparison to other radiolabelled CCK-B analogues.
We conclude that the new prochelator 6d has widespread utility in the field of metallo-radiopeptides, other radiolabeled biomolecules and for the synthesis of Gd^{3+} based MRI contrast agents[9]. DOTAGA will allow to label with different radiometals for both diagnostic (^{111}In, $^{67/68}$Ga) and internal radiotherapeutic applications (^{90}Y, ^{177}Lu).

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2.Parker, D. *Chem. Soc. Rev.* 1990, *19*, 271-291
3.Heppeler, A.; Froidevaux, S.; Mäcke, H. R.; Jermann, E.; Behe, M.; Powell, P.; Hennig, H. *Chem. Eur. J.* 1999, *5*, 1974-1981.
4.André, J. P.; Mäcke, H. R.; Tóth, É.; Merbach, A. A. *JBIC* 1999, *4*, 341-347.
5.Kruper, W. J.; Rudolf, P. R.; Langhoff, C. A. *J. Org. Chem.* 1993, *58*, 3869-3876.
6.Holmberg, C. *Chemische Berichte* 1927, *60*, 2197, 2205.
7.Armstrong, A.; Brackenridge, I.; Jackson, R. R. W.; Kirk, J. M. *Tetrahedron Lett.* 1988, *29*, 2483-2486.
8.Typical procedure for the reaction of 1 to 2: To a solution of 6 g (25.9 mmol) L-glutamic acid-5-benzylester(1d) and 9.1 g (88.5 mmol) sodium bromide in 45ml aqueous 1N hydrobromic acid (46 mmol) cooled to 0°C was added portionwise 3.175 g (46 mmol) sodium nitrite. After stirring for 2h at 0°C 2.25 ml conc. sulfuric acid was added followed by diethylether. The water phase was extracted 3 times with diethylether. The combined organic phases were extracted 4 times with brine, dried over Na_2SO_4 and concentrated. The crude product was purified by chromatography (silica gel 60;

hexane/EtOAc 3:1 to 2:1) and obtained as a yellow oil in a yield of 4.8 g (63%). ^{1}H-NMR (300 MHz, $CDCl_3$, $SiMe_4$): 10.1 (1H, COOH); 7.3 (m, 5H, Ar); 5.15 (s, 2 H, CH_2-Ph); 4.4 (dd, ^3J = 5.7, 1H, CHBr); 2.6 (t, ^3J = 6.8, 2H, CH_2-COOBzl); 2.5-2.2 (m, 2H, CHBr-CH_2-CH_2); ^{13}C-NMR (75 MHZ, $CDCl_3$, $SiMe_4$): 174.5 (COOH); 171.9 (COOBzl); 135.5 (CH_2C(Ar)); 128.6, 128.4, 128.3 (C(Ar)); 66.8 (O-CH_2-Ar); 44.1 (HCBr); 31.4 (HCBr-CH_2); 29.4 (CH_2COOBzl; EI-MS m/z (intensity): 302, 300 (12, $[M]^+$); 91 (100, $[Bzl]^+$).

Reaction of 2 to 3: To a solution of 4.8 g (15.9 mmol) 2d in 20 ml $CHCl_3$ a solution of 6.26 ml (34.1mmol) TBTA (*tert*-butyltrichloroacetimidate) in 20 ml cyclohexane was added dropwise over 20 min. During the addition a white precipitate formed, which was dissolved by the addition of 3.5 ml of DMA followed by 320 µl boron trifluoride ethyl etherate as catalyst. The reaction mixture was stirred for 3 d at RT. The mixture was concentrated and the remaining DMA phase was extracted 3 times with 30 ml hexane. The hexane phase was evaporated and the residue chromatographed over silica gel 60 (Hexane/EtOAc 20:1 later 9:1) affording 3.5 g (61%) of a colourless liquid. ^{1}H-NMR (300 MHz, $CDCl_3$, $SiMe_4$): 7.4 (m, 5H, Ar); 5.15 (s, 2H, CH_2-Ph); 4.35 (dd, 1H, CHBr); 2.6 (td, 2H, CH_2-COOBzl); 2.5-2.2 (m, 2H, CHBr-CH_2-CH_2); 1.5 (s, 9H, $C(CH_3)_3$). ^{13}C-NMR (75 MHZ, $CDCl_3$, $SiMe_4$): 172.4 (COOBzl); 168.7 (COOtBu); 136.1 (CH_2C(Ar)); 129.0, 128.8, 128.7 (C(Ar)); 83.1 ($C(CH_3)_3$); 67.0 (O-CH_2-Ar); 47.1 (HCBr); 32.0 (HCBr-CH_2); 30.1 (CH_2COOBzl); 28.1 ($C(CH_3)_3$); EI-MS m/z (intensity): 302, 300 (18, $[M-C_4H_9]^+$); 57 (100, $[C_4H_9]^+$).

9.André, J. P.; Tóth, É.; Fischer, H.; Seelig, A.; Mäcke, H. R.; Merbach, A. A. *Chem. Eur. J.* **1999**, *5*, 2977-2982

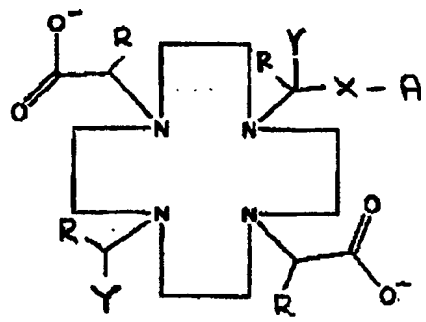
10.General procedure of the monoalkylation of cyclen: A solution of 870 mg (2.44 mmol) α-bromoglutaric acid-1-*tert*-butylester-5-benzylester(3d) in $CHCl_3$ was added dropwise over a period of 1h to a solution of 885 mg (4.9 mmol) cyclen in 4ml $CHCl_3$. The mixture was stirred for 2 d at room temperature and concentrated to a brown oil. The crude product was purified by column chromatography (silica gel 60; ethanol/ NH_3 95:5), yield 920 mg (83%) of a colourless oil. ^{1}H-NMR (300 MHz, $CDCl_3$, $SiMe_4$): 7.35 (m, 5H, Ar); 5.1 (s, 2H, CH_2-Ph); 3.25 (dd, 1H, CHBr); 2.9-2.5 (m, 18H, NCH_2, CH_2COOBzl); 2.2-1.85 (m, 2H, CHN-CH_2-CH_2); 1.45 (s, 9H, $C(CH_3)_3$); ^{13}C-NMR (75 MHZ, $CDCl_3$, $SiMe_4$): 173.1 (COOBzl); 171.5 (COOtBu); 135.8 (CH_2C(Ar)); 128.5, 128.3, 128.2 (C(Ar)); 81.4 ($C(CH_3)_3$); 66.2 (O-CH_2-Ar); 63.5 (HCNCH$_2$); 48.8, 48.0, 46.5, 45.6 (NCH_2CH_2N); 30.6 (CH_2COOBzl); 28.2 ($C(CH_3)_3$); 24.5 (HCN-CH_2); EI-MS m/z: (intensity): 449.3 (56, $[M+H]^+$); 245.8 (100, $[M+ CH_3CN+2H]^{++}$). Synthesis of 1-(1-carbobenzyloxy-3-carbo*tert*butoxypropyl)-4,7,10-(carbo*tert*butoxymethyl)-1,4,7,10-tetraazacyclododecane (5d): A suspension of 1.1g (5.6 mmol) bromoacetic acid-*tert*-butylester, 1.02 g (2.27 mmol) 1-(1-carbobenzyloxy-3-carbo*tert*butoxypropyl)-1,4,7,10-tetraazacyclododecane (4d), and 2.63 g (19.1 mmol) of dry potassium carbonate in 10 ml dry acetonitrile was stirred for 18 h at rt and was filtrated afterwards over Celite and evaporated to dryness. The crude product was purified by column chromatographie (silica gel 60; CH_2Cl_2/EtOH 9:1 followed by EtOH/NH_3 95:5) yield 1.3 g (73%) of a yellow oil(5d). ^{1}H-NMR (300 MHz, $CDCl_3$, $SiMe_4$): 7.35 (m, 5H, Ar); 5.1 (s, 2H, CH_2-Ph); 3.6-1.9 (m, 27H, CHN, NCH_2, CH_2COOBzl, CHN-CH_2-CH_2, CH_2COOC$(CH_3)_3$); 1.45 (s, 36H, $C(CH_3)_3$); ^{13}C-NMR (75 MHZ, $CDCl_3$, $SiMe_4$): 174.6 (COOBzl); 172.9, 172.8, 172.6 (COOtBu); 135.6 (CH_2C(Ar)); 128.5, 128.3, 128.2 (C(Ar)); 82.4, 81.8, 81.8 ($C(CH_3)_3$); 66.3 (O-CH_2-Ar); 55.8, 55.7, 55.4, 52.6, 52.3, 50.3, 48.5, 48.1, 47.1, 44.3 (13C, HCNCH$_2$, NCH_2CH_2N, CH_2COOtBu, CH_2COOBzl); ($NCHCH_2CH_2$); 28.0, 28.0, 27.8, 27.6 ($C(CH_3)_3$); EI-MS m/z (intensity): 813.6 (22, $[M+Na]^+$); 791.6 (38, $[M+H]^+$); 396.5 (100, $[M+2H]^{++}$).

Synthesis of DOTAGA(tBu)$_4$(6d): 600 mg (0.76 mmol) 5d was dissolved in methanol, and 30 mg Pd/C suspended in 1 ml H_2O was added. The mixture was hydrogenated for 2 d, filtrated over Celite and evaporated to dryness. The crude product was chromatographed on silica gel 60 (EtOH/NH_3 95:5) to obtain 470 mg (84.6%) of a white solid (6d). ^{1}H-NMR (300 MHz, $CDCl_3$, $SiMe_4$): 6.5 (br, 1H, COOH); 3.6-2.0 (m, 27H, CHN, NCH_2, CH_2COOH, CHN-CH_2-CH_2, CH_2COOC$(CH_3)_3$); 1.45 (s, 36H,

$C(CH_3)_3$); ^{13}C-NMR (75 MHZ, $CDCl_3$, $SiMe_4$): 175.2 (<u>C</u>OOH); 175.0, 172.9, 172.8, 172.6 (<u>C</u>OOtBu); 82.4, 82.1, 81.9 (<u>C</u>(CH_3)$_3$); 55.8, 60.1 (N<u>C</u>HCOOtBu); 55.9, 55.8, 55.6, 52.7, 52.6, 52.5, 48.6, 48.5, 48.2, 47.1, 44.3 (12C, N<u>C</u>H_2<u>C</u>H_2N, <u>C</u>H_2COOtBu, <u>C</u>H_2COOH); 33.4 (NCH<u>C</u>H_2<u>C</u>H_2); 27.9, 27.8 (C(<u>C</u>H_3)$_3$); EI-MS m/z (intensity): 723.5 (27, $[M+Na]^+$); 701.5 (68, $[M+H]^+$); 351.4 (100, $[M+2H]^{++}$).

11.Data of DOTAGA-CCK-B analogue (DOTAGA-7): Yield: 12.7 mg, HPLC purity >95%, (+) EI-MS m/z (intensity): 1486.1 (48, $[M+H]^+$); 743.7 (60, $[M+2H]^{++}$); (-) EI-MS m/z (intensity): 1484.0 (28, $[M+H]^-$); 741.8 (90, $[M+2H]^{--}$)

The invention relates also to molecules for radioactive labelling which molecules have the general formula I:



(I)

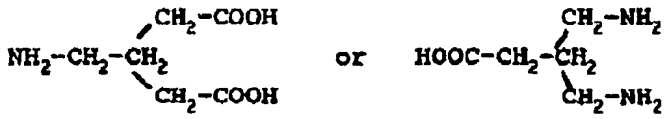
in which:

both Y groups may be positioned either trans as shown or cis;

A is an effector molecule, such as a peptide, in particular octreotide, CCK, substance P, gastrine, a protein, in particular an antibody or enzyme, sugars or radiosensitizing agents, like doxorubicin;

R is a hydrogen, a C_1-C_3 alkyl or a alcohol;

X is a spacer, in particular $(CH_2)_n$-X', in which n is 1-10 and X' is COOH, NH_2, SH, OH or O-halogen, in which halogen is in particular Br, I or Cl
or a molecule of the formula



Y is COO^-, CH_2CONH_2, CH_2CH_2OH.

It has been found that when using the prochelators of the invention for preparing biologically active molecules, these molecules have better biological properties than molecules prepared with other (pro)chelators. The advantages for example are a better labelling yield according to the following table 2:

Table 2

	amount of radioactive label	labellingefficiencyof DOTATOC-peptide	labellingefficiency of DOTA3-peptide (DOTAGA) (invention
5 µg of the compound to be labelled	5 mCi ^{90}Y	99.5%	99.9%
	10 mCi ^{90}Y	95%	100%
	20 mCi ^{90}Y	92%	99.9%

In addition, the satbility is higher. The reaction of ^{90}Y-DOTATOC or ^{90}Y-DOTAGA at 37°C with the chelator DTPA results in ^{90}Y-DTPA. The halflife of this reaction is 23 hours for DOTATOC and 79 hours for DOTAGA.

Table 3 shows various biological properties of the compound of the invention Y-DOTA3TOC and Y-DOTAta.13TOC. The radiometal is not shown.

The figures 1 and 2 show synthetic routes for compounds of the invention.

Biological results of Yttrium labelled Peptides

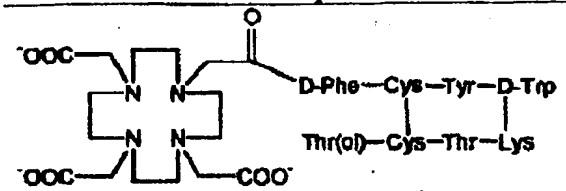
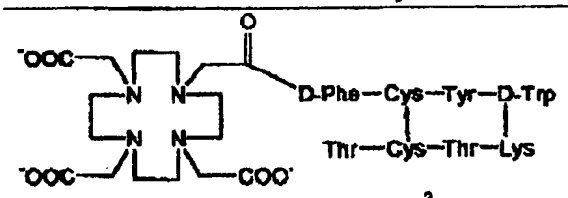
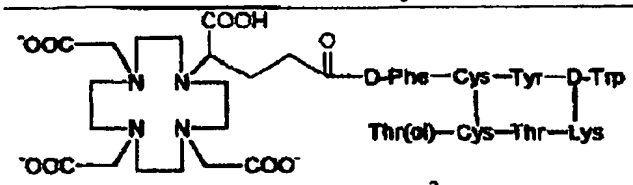
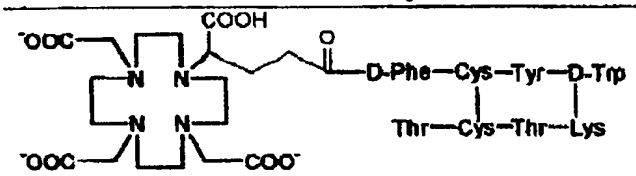
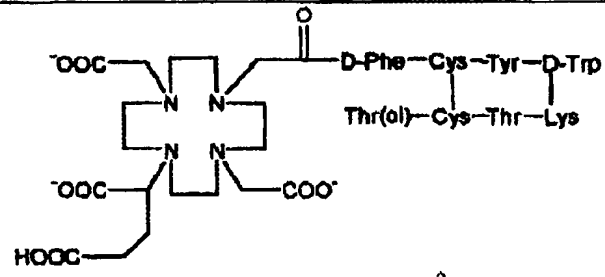
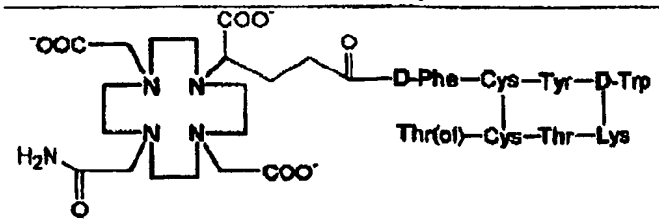
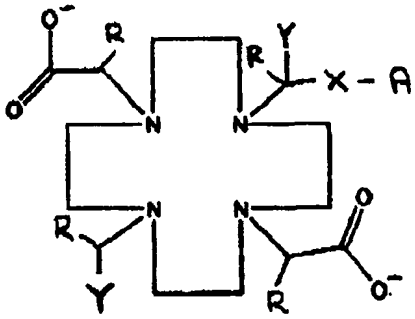
Peptide	IC_{50}(hsst2)	Tumor	Kidney	Charge
 Y-DOTATOC: Y-DOTAtyr^{3}octreotide	11 ± 1.7	13.5	12.3	+1
 Y-DOTATATE: Y-DOTAtyr^{3}octreotate	1.6 ± 0.4	14.5	8	0
 Y-DOTA3TOC: Y-DOTA3tyr^{3}octreotide	1.5 ± 0.5	30	56	0
 Y-DOTA3TATE: Y-DOTA3tyr^{3}octreotate	35	13.5	68	-1
 Y-DOTAt3TOC: Y-DOTAt3tyr^{3}octreotide	28 + 10	23.5	25.5	0
 Y-DOTAta.13TOC Y- DOTAta.13tyr^{3}octreotide	0.23			+1

Table 3

CLAIM

Biologically active molecules for radiometal labelling having the general formula I:



(I)

in which:

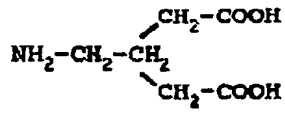
both Y groups may be positioned either trans as shown or cis;

A is an effector molecule, such as a peptide, in particular octreotide, CCK, substance P, gastrine, a protein, in particular an antibody or enzyme, sugars or radiosensitizing agents, like doxorubicin;

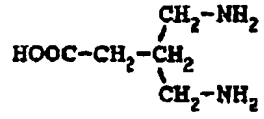
R is a hydrogen, a C_1-C_3 alkyl or a alcohol;

X is a spacer, in particular $(CH_2)_n$-X', in which n is 1-10 and X' is COOH, NH_2, SH, OH or O-halogen, in which halogen is in particular Br, I or Cl

or a molecule of the formula



or of the formula



Y is COO^-, CH_2CONH_2, CH_2CH_2OH.

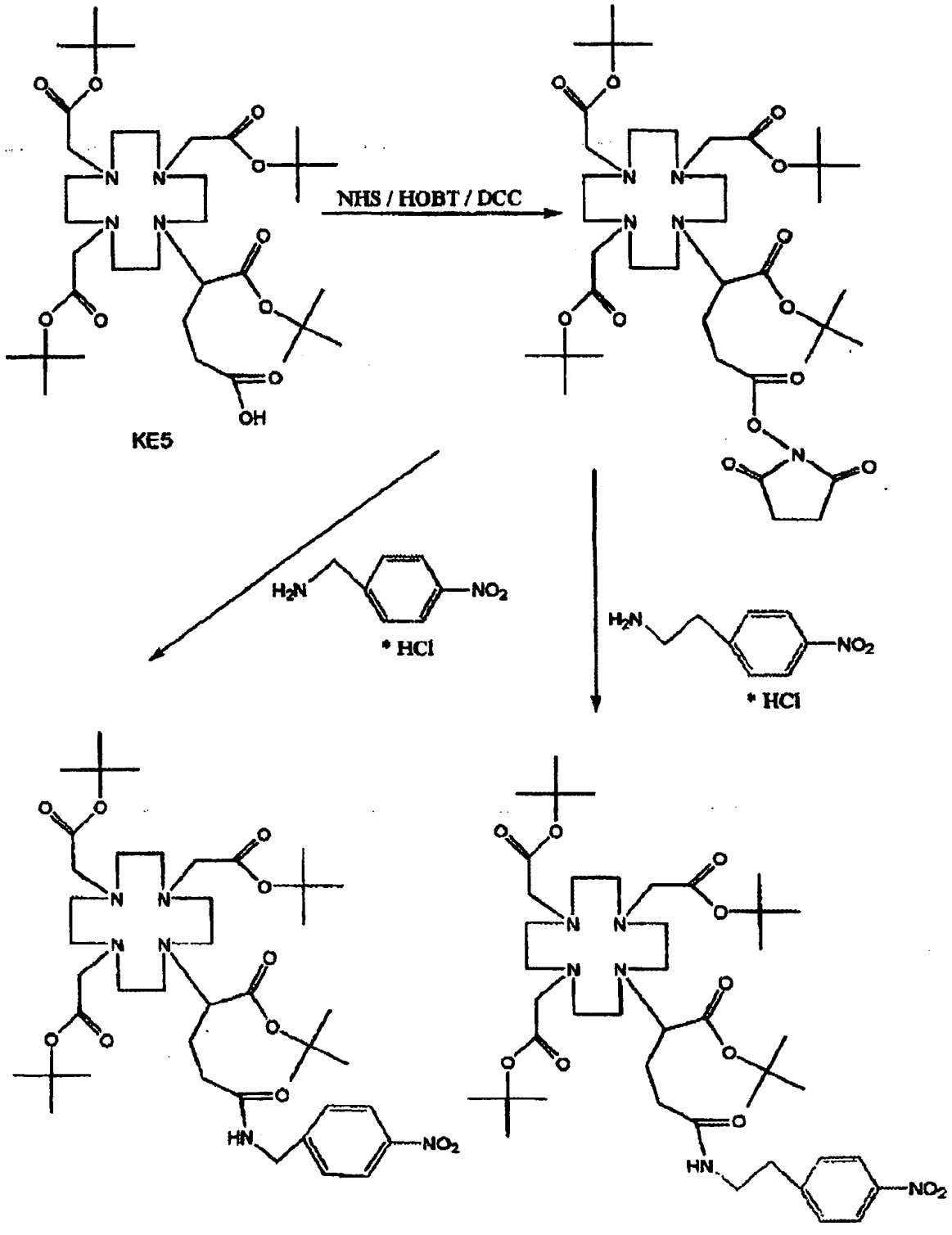
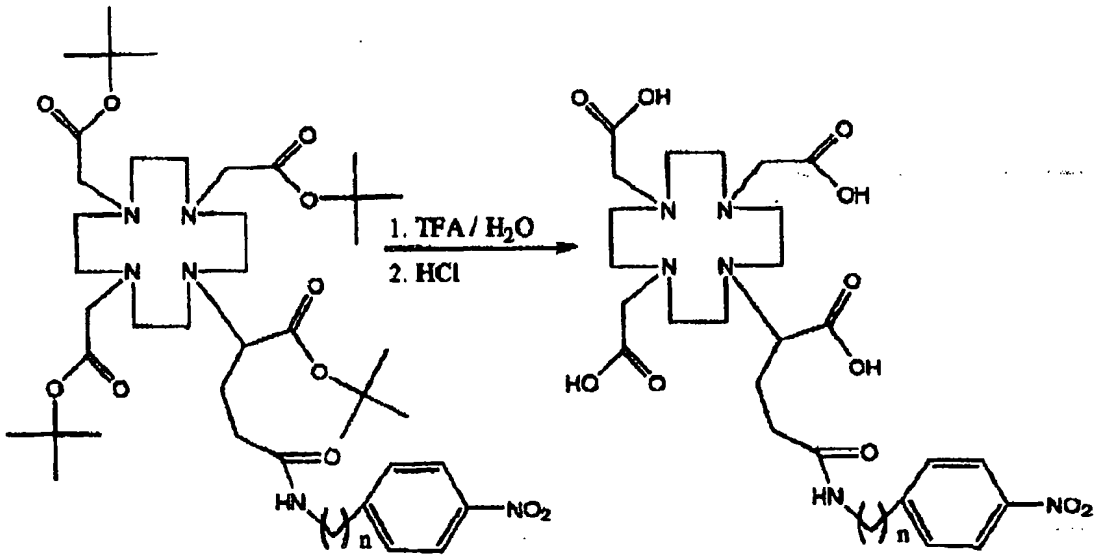
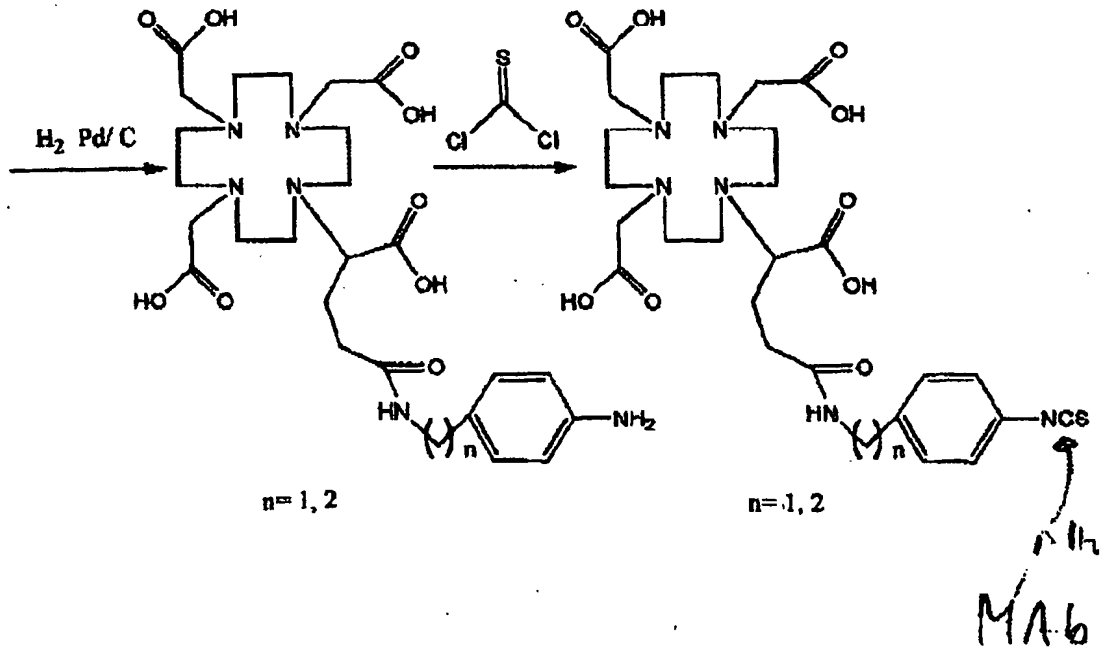


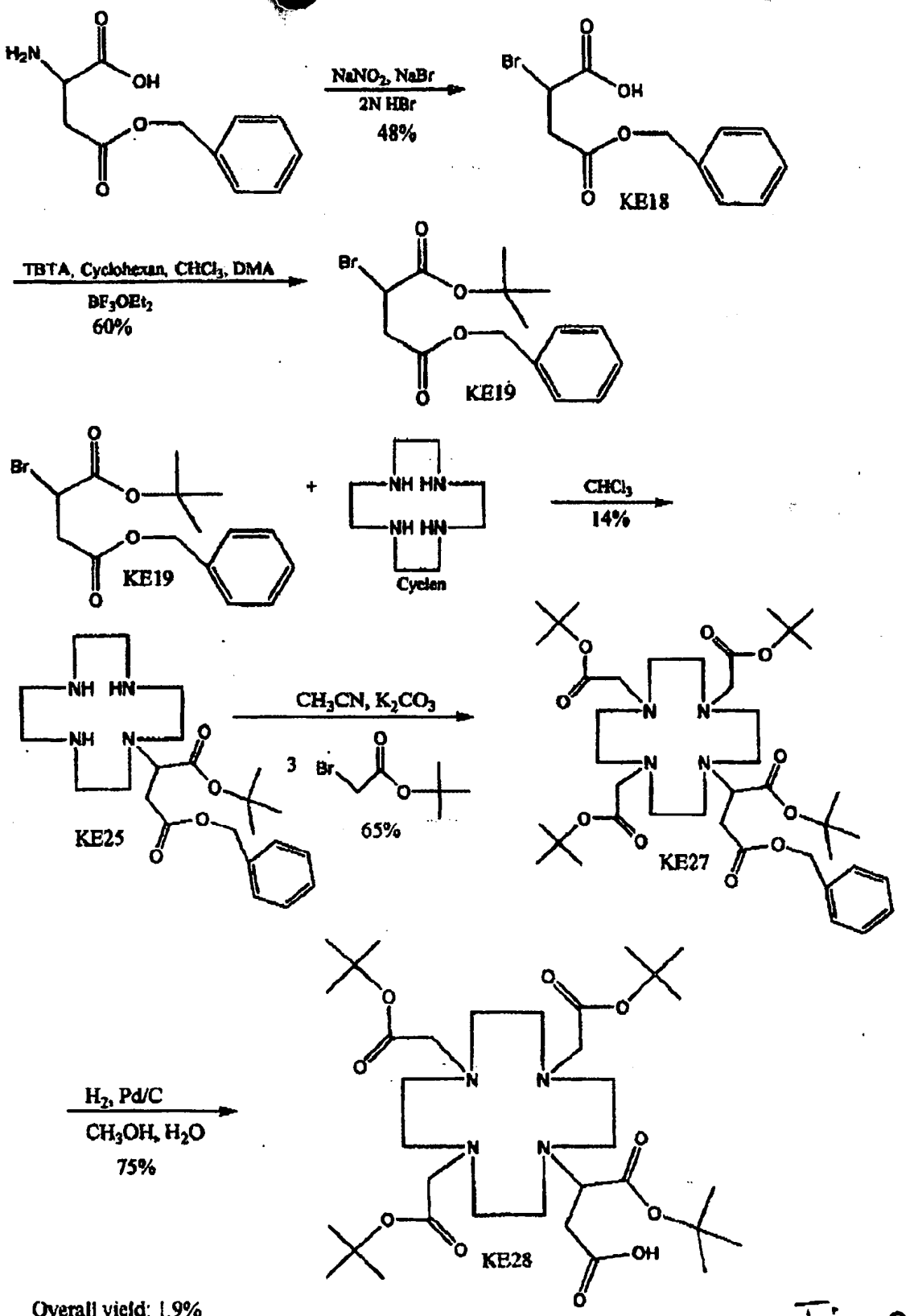
Fig. 1-A





MA-6

Fig. 1-B



Overall yield: 1.9%

Fig.2

ADDITIONAL REPRESENTATIVES

Hoorweg, Petrus Nicolaas
Schumann, Bernard Herman Johan
't Jong, Bastiaan Jacobus
De Ranitz', Remco Engbert Pieter
Prins, Hendrik Willem
Land, Addick Adrianus Gosling
Louët Feisser, Arnold
Hootveld, Arjen Jan Winfried
Bruin, Cornelis Willem
Konings, Lucien Marie Cornelis Joseph
Eveleens Maarse, Pieter
Verhaag', Peter Paul Johannes Marie
Bartelds, Erik
Van Someren, Petronella Franciscа Hendrika Maria
Duxbury, Stephen
Van Wijngaarden', Marcus Antonius Alexander
Grootscholten, Johannes Anthonius Maria

'Representative acc. art. 134(7) EPC